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Change in alkaline phosphatase isoenzyme pattern in urine as possible marker for renal disease

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Change in alkaline phosphatase isoenzyme pattern in urine as possible marker of renal disease. The human kidney contains two types of alkaline phosphatase (AP) isoenzymes: a hepatic type of AP and an intestinal-like AP. Intestinal-like AP, measured by immunotitration techniques, is a minor component (1 to 4%) of the total AP activity. It is found only in the particle-free fraction (cytoplasm) and is located, with immunofluorescent techniques, in some of the proximal convoluted tubules. Urinary AP activity is found after high-speed centrifugation in the supernatant (×100,000*g*), as well as in the sediment, and may be extracted from the sediment after solubilization with n-butanol. Both types of these renal isoenzymes contribute to urinary AP activity. Biochemical characterization (effect of inhibitors, thermostability, denaturing with urea, and so on) revealed that urinary intestinal-like AP and renal intestinal-like AP are identical. Both, however, have been distinguished as multiple forms of AP from the small intestine. Most of the urinary AP activity of healthy persons (22 volunteers) was found in the sediment and consisted of liver-type AP. Urinary AP of patients with diseases, after application of potentially nephrotoxic drugs or during rejection episodes of renal allografts, contains little sediment activity, but it contains increased amounts of urinary intestinal-like AP.

Modification de l'aspect des isoenzymes de la phosphatase alkaline (AP) dans l'urine comme marqueur possible d'une affection rénale. Le rein humain contient deux types d'isoenzymes de l'AP: le type hépatique et un type semblable à l'enzyme intestinale. L'AP intestinale déterminé par immunotitration, est un composant mineur (1 à 4%) de l'activité totale de AP. L'AP intestinale est trouvé seulement dans la fraction cytoplasmique et localisé dans quelques convolutions proximales comme le montre l'immunofluorescence. L'activité AP de l'urine est observée après centrifugation à grande vitesse dans le surnageant ×100,000*g* de même que dans le sédiment et peut être extraite du sédiment par solubilisation dans le n-butanol. Les deux types d'isoenzymes observés dans le rein contribuent à l'activité AP de l'urine. La caractérisation biochimique (effet des inhibiteurs, thermostabilité, dénaturation par l'urlée, etc.) révèle que l'AP intestinale de l'urine et l'AP intestinale de le rein sont identiques. Cependant, ces deux formes ont été reconnues comme des formes multiples de l'AP intestinale isolée de l'intestin grêle. La plus grande partie de l'activité urinaire de l'AP chez des sujets sains (22 volontaires) a été découverte dans le sédiment et consistait en AP de type hépatique. L'AP urinaire de malades, aprés administration de drogues néphrotoxiques, au cours d'épisodes aigus de rejet, contenait peu d'activité sédimentable mais des quantités élevées de l'AP intestinale urinaire.

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Previous investigations [1–3] presented evidence that the mammalian and human kidney contains two antigenically distinct isoenzymes of alkaline phosphatase (AP): one is a liver type (K_L) and the other an intestinal type (K_I). Alkaline phosphatase activity (orthophosphoric monoester phosphohydrolase [alkaline optimum], E.C. 3.1.3.1) in rat kidney tissue was localized in the microvilli of the proximal tubules [4]. So far, the differentiation of these isoenzymes has not been made in the kidney regarding their ultrastructural localization and biochemical function.

Butterworth et al [5] and recently Korngold [6] described the urinary AP as having a lower molecular weight compared with kidney AP and as reacting with an intestinal AP antiserum. But because their studies were concentrated primarily on urinary AP of patients with proteinurea or acute tubular necrosis, no comparison to the urinary form of healthy persons could be drawn.

Because the immunotitration technique was found to be a suitable tool for investigating the isoenzyme pattern of various enzymes in blood or tissue [7, 8], we used this method to study the alkaline phosphatase isoenzymes of kidney and urine so that we could compare the output of these various isoenzymes under normal and pathologic conditions. Furthermore, we extended our investigations to the influence of the aminoglycosides on the AP isoenzyme pattern in kidney and urine. The nephrotoxicity of aminoglycosides is a known fact [9–11],

and the determination of the daily output of membrane-bound enzymes, such as alanine aminopeptidase, as a more sensitive indicator of the renal damage, has reconfirmed this.

Methods

Enzyme assay. Alkaline phosphatase activity was measured, by the method of Hausamen et al [12], at 25° C with 10 mM *p*-nitrophenylphosphate as the substrate in 1 M diethanolamine and hydrochloric acid buffer (pH, 9.8) in a final volume of 1 ml, containing 0.5 mM magnesium chloride. Enzyme samples were preincubated for at least 15 min in the test buffer. The reaction was started by adding the substrate. Absorbance change was measured at 405 nm in an Eppendorf photometer (ϵ = 18.2 $cm^2/\mu mole$). Creatine kinase was tested by the *N*-acetylcysteine-activated method of Oliver [13]; lactate dehydrogenase activities, by the standard method; and alanine aminopeptidase, by a previously described method [14]. Enzyme activities are given as international units.

Preparation of antibodies. Rabbit antisera against purified AP isolated from calf intestinal mucosa and human kidney were produced by the method described recently [2]. They were purified on protein A [15] without significant loss of immunologic activity, as already described [14].

Cell fractionation was done in 0.25 M sucrose by differential centrifugation, as described by Schneider [16]. The cytoplasmatic fraction (×18,000*g*) had been further purified from membranous particles by ultracentrifugation (×100,000*g*, 60 min).

Butanol extraction of membrane-bound AP activity in urine and tissue homogenates was done with a threefold amount of 30% n-butanol and 70% Tris hydrochloric acid (10 mM; pH, 7.5) containing 2 mM magnesium chloride and 0.025 mM zinc chloride (standard buffer). Tissues were homogenated with a Potter-Elvehjem. The mixture was allowed to stand first at room temperature for 12 hours; afterwards it stood in the cold for 12 hours, continuously being stirred. After centrifugation, the solution was dialyzed against standard buffer.

Immunotitration assay. For our routine precipitation test, we usually incubated 10 mU of AP in a standard buffer solution containing 2.5% polyethylene glykol and 0.2% of bovine serum albumin with increasing amounts of antiserum either against kidney AP or intestine AP in a total volume of 200 μl. Addition of bovine serum albumin has been found suitable to stabilize enzymatic activity under present conditions. The incubation mixture was incubated for 1 hour at 37° C, and afterwards it was kept in the cold overnight. After it was centrifuged at high speed (30 min, ×48,000*g*), we estimated the difference between the residual activity in the supernatant and the blank without antiserum. The difference revealed the actual amount of the various isoenzymes. Knowing the titers of our applied antisera and for simplifying this method, we just determined the final point of the titration curve by incubating the assay with an excess of antiserum. One complication occurred when we compared the immunotitration technique for either human tissue extract with that for AP in urine. We needed more antiserum, in the case of urine AP, to get the quantitative precipitation. Urine samples were concentrated up to $^1/_{25}$ of the initial volume and ultracentrifuged (×100,000*g*, 60 min).

Separation of AP isoenzymes by affinity chromatography. Ultracentrifuged tissue homogenates and urinary samples were dialyzed against 10 mM Tris hydrochloric acid (pH, 8.0). Affinity chromatography on L-histidyl-diazobenzylphosphonic acid agarose was carried out as described by Landt, Boltz, and Butler [17]. The eluted enzymes were tested by immunotitration for their antigenetic purity.

Electrophoresis on cellulose acetate stripes was carried out in Boskamp chambers, with 48 mM Tris barbituric acid (pH, 8.6). With a voltage of 200 V, electrophoresis was carried out for 2 hours. Enzymatic staining of AP was performed with α-naphthylphosphate [2] as substrate and with Fast blue BB salt as the coupling dye component, as described [18].

Molecular weight was determined with a Beckman ultracentrifuge and polymer tubes that contained 4 ml of standard buffer. Sucrose gradient was obtained by the overlaying of 0.5 ml of 20% sucrose-Tris buffer solution by 0.5-ml portions with decreasing contents of sucrose up to a final concentration of 5% sucrose. The gradient was allowed to develop by standing for 4 to 6 hours. The sample was applied together with creatine kinase, lactate dehydrogenase, and hemoglobin above the gradient solution in a volume of 20 to 50 μl by an Eppendorf pipette. The centrifuge was run for 20 hours. All tubes were eluted by fractioning into 23 fractions. Creatine kinase and lactate dehydrogenase activities were tested, and the distance of hemoglobin from the start was measured. Molecular weights were determined by the equations of Martin [20].

Molecular weight by gel filtration was determined with a Sephacryl S-200 superfine column (140 × 1.5 cm) in buffer, containing 0.2 M sodium chloride to

avoid any ionic interaction. Lactate dehydrogenase, creatine kinase, and bovine serum albumin were used as standards; void volume was determined by Dextran-blue.

Inhibition studies were carried out by incubating the enzyme with L-phenyl-alanine for 15 min in a usual test buffer [20]. Heat stability was tested by incubating the enzyme diluted in standard buffer for 90 min at 56° C, cooling it immediately afterwards, testing it as usual, and comparing it with the same enzyme dilution that was not heated before and tested in the same way.

Inactivation test in 5 M urea was carried out in the test buffer by incubating the sample for 90 min. An aliquot was incubated for the same time in the test buffer without urea and was taken to have 100% residual activity.

Immunofluorescence. For localizing the intestinal AP isoenzyme in the human renal tissue, we used 7-μm thick cryostat sections from nitrogen-frozen blocks of fresh, normal, cadaveric kidney. In the two-layer (indirect) staining method, the unfixed sections were incubated for 20 min at 37° C in a damp chamber with serial dilutions of the rabbit antiserum (as IgG) against intestinal AP isoenzyme. The incubation was followed by three-fold careful washing in PBS and a second incubation under the same conditions with 1:8 diluted FITC-labeled anti-rabbit goat antiserum (Miles). The washed sections were examined under a Universal Microscope Zeiss with a mercury vapour burner and a cardioid condensor. The exciting filter used was BG 12/3 (green exciting), and the barrier filter was GG9. The same sections, incubated with only FITC-labeled anti-rabbit antiserum, others with rabbit antiserum anti-intestinal AP by itself, were used as controls. The controls showed no fluorescent staining.

Results

Distribution of AP isoenzymes in urine. Ultracentrifugation of urine samples of healthy persons revealed that only about 35% of total AP activity is found in the particle-free fraction ($\times$100,000*g* supernatant) (Table 1). The relative amount of soluble AP increased in a significant way in the urine of patients with renal disease, such as pyelonephritis or glomerulonephritis, and after kidney transplantation, but also in the urine of healthy persons after aminoglycosides-treatment. Comparing the daily output of AP of such pathologic urine with that in normal urine, it was obvious that this change in the relative amount of sedimentable AP activity was not due to an increase of enzymatic activity.

While investigating AP isoenzyme patterns in urine by immunotitration technique, we found two isoenzymes present in urine: a "liver-like" fraction (U_L) and an "intestinal-like" one (U_I). The AP activity of the *sediment* obtained by ultracentrifugation consisted of approximately 90% and more of U_L. Only in a few samples, small amounts of up to 10% of U_I were found.

The urinary AP activity found in the *supernatant* contained intestinal AP in an amount of about 50% for healthy persons and of 70 to 80% for patients

Table 1. Distribution of alkaline phosphatase (AP) isoenzymes in human urine[a]

Diagnosis	AP	AP	AP in particle-free fraction		Isoenzyme pattern of total AP[b]			
					U_I		U_L	
	U/liter	*mU/24 hr*	%	*mU/24 hr*	%	*mU/24 hr*	%	*mU/24 hr*
Control (22 individuals)	3.5	5829	35	2040	19	1108	71	4139
Range	(2.2 to 5.6)	(3950 to 7980)	(13 to 53)	(416 to 2892)	(9 to 29)	(589 to 1518)	(47 to 80)	(2447 to 5158)
	5.6)							
Chronic GN	3.8	6878	46	3168	15	1045	83	5706
	2.1	683	78	532	64	436	36	243
GN	5.7	5244	56	2937	26	1380	65	3423
	5.0	5082	90	4574	69	3506	10	508
Post GN	2.4	1632	54	881	14	231	78	1273
Chronic PN	3.4	8432	71	5987	66	5574	33	2790
	1.5	1680	77	1294	59	996	41	684
	6.8	11696	74	8655	52	6128	45	5276
Nephrotic syndrome	2.8	10416	61	6353	50	5216	53	5557
	8.7	6160	87	5359	47	2880	58	3572
Suspicion of hydronephrosis	4.6	8004	48	3842	39	3130	63	5036
Renal insufficiency	3.5	5460	88	4805	61	3324	37	2040

[a] Abbreviations are defined as: GN, glomerulonephritis; PN, pyelonephritis; U_I, intestine-like AP in urine and; U_L, liver-like AP in urine.

[b] Determined by immunotitration.

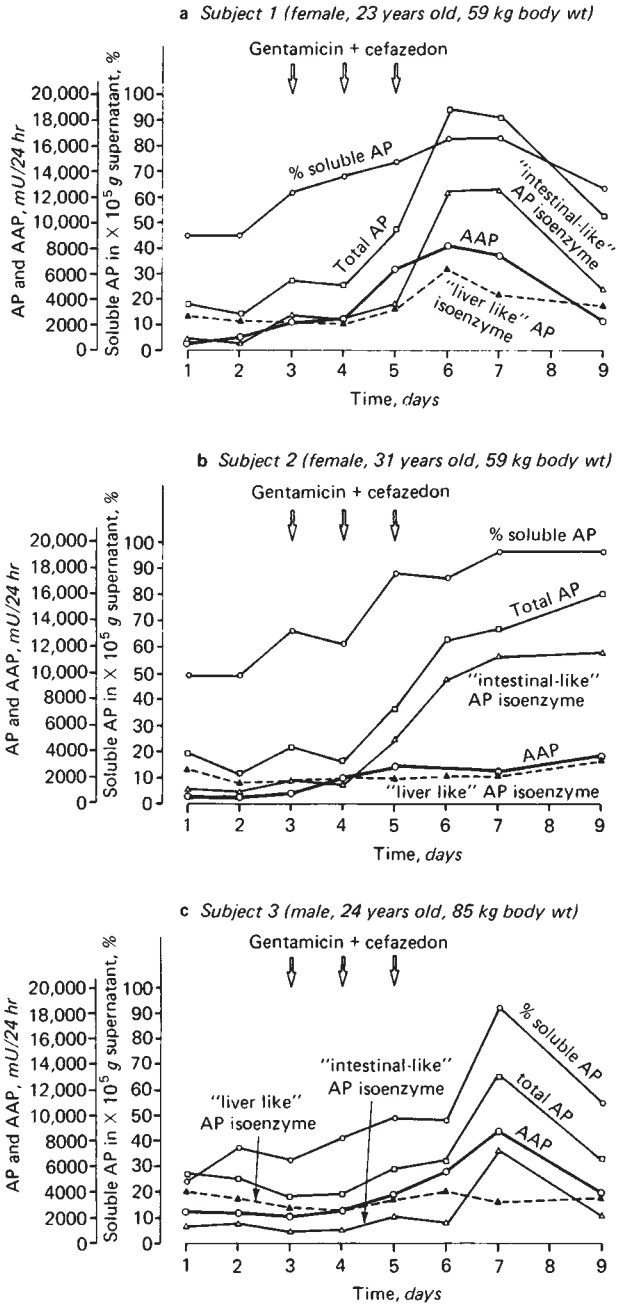

Fig. 1 a to c. *Mean urinary level of alkaline phosphatase (AP), alanine aminopeptidase (AAP) activity, and isoenzyme pattern of AP before, during, and after i.m. administration of gentamicin (3 mg/kg) and cefazedon (6 g) on 3 consecutive days.*

with renal disease. The rest of the AP activity was attributed to the liver-like isoenzyme. Based on the change of solubility, therefore, the total urinary AP from healthy persons contained about 19% intestinal-like isoenzyme (U_I), whereas in the urine from patients with renal disease this form increased to about 50 to 60% of the total activity (Table 1).

The application of a combination of cefazedon and gentamicin to healthy persons caused a nearly fivefold increase of AP activity in the urine, parallel to an increase of soluble enzyme up to 80 to 90% from day 2 after the first application, with a maximum of 2 days or even later after the end of the application. The isoenzyme pattern changed in such a way that U_I rose after application while U_L remained nearly constant (Fig. 1, a to c). Series of urine samples of one person after kidney transplantation were tested for AP activity, sedimentation property, and isoenzyme pattern. We found a fourfold increase of urinary AP activity. The maximum was reached on day 11 after the transplantation due to an increse of U_I in urine, going up from approximately 3000 mU/24 hours immediately after transplantation to 27,000 mU/24 hours on day 11. Alanine aminopeptidase, which is regarded as a sign of rejection crisis, reached its maximum on day 9 (Fig. 2).

Amount and localization of K_I in human and bovine kidney. So far, human kidneys investigated for AP isoenzyme pattern exhibited the presence of 1 to 4% K_I ("intestinal-like") of the total activity besides the K_L ("liver-like") AP. Cell fractionation studies were carried out with bovine kidney, which contains the same amount of intestinal-like AP as does the human kidney, by the differential centrifugation method of Schneider [16]. They were resected immediately after death and used for experiment on the same day without freezing. These conditions were necessary to avoid cellular destruction and could not be fulfilled by human tissue. These experiments revealed that K_I is located only in the cytosol (Table 2). No membrane-bound K_I was found.

When the tissue of the human kidney was cut into five sections, from the surface to the interior of the organ, K_I was found in the cortex (Fig. 3), whereas no K_I was located in the medulla. To obtain further information about the morphologic origin of K_I, we carried out immunofluorescence studies on human kidney with purified antiintestinal AP serum. The renal cortex showed fluorescence in about 10% of all observed convoluted tubules. No staining was remarked in the glomeruli and medulla (Fig. 4).

Biochemical characterization of intestinal-type AP (human) isolated from intestine, kidney (K_I), and urine (U_I). Homogenates of intestinal and kidney tissues, as well as of urine, usually contain both isoenzymes of AP, liver-type and intestinal-type, in various amounts. To achieve enriched isoenzyme preparations, we carried out affinity chromatography with L-histidyldiazobenzyl phosphonic acid

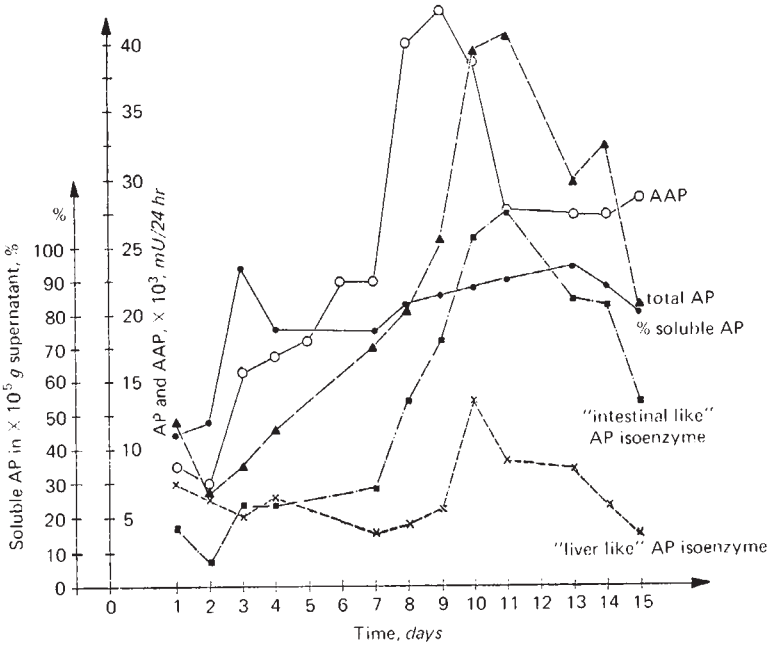

Fig. 2. *Mean urinary level of alkaline phosphatase (AP), alanine aminopeptidase (AAP) activity, and AP isoenzyme pattern after kidney transplantation.*

agarose [17]. As Mössner and Pfleiderer have found (unpublished results), only intestinal-like AP (free of neuraminic acid) was bound by means of this technique. Therefore, it was possible to separate K_I completely from K_L. About 1 U of each form had been prepared from crude kidney extract or urine. As proven by immunotitration technique, none of these fractions contained even a small amount of the liver-like isoenzyme.

Immunologic studies could demonstrate that K_I and U_I antigens are precipitated, as well as intestinal AP, by using anticalf intestinal AP serum as antibody. No remarkable difference in its titer against these antigens could be detected. Although affinity of antibodies used in these experiments was different against human and bovine antigens, they showed good cross-reactivity. So they could be used in the experiments described above. Determination of the molecular weight with ultracentrifugation in sucrose density gradient exhibited 124,000 daltons for all three forms. These findings were in contrast to results achieved by gel filtration experiments on a Sephacryl S-200 superfine column, indicating a lower molecular weight of 80,000 daltons for U_I, compared with 120,000 to 130,000 daltons for intestinal AP found by using the same technique.

Table 2. Cellular distribution of alkaline phosphatase (AP) in bovine kidney, estimated by means of fractionated centrifugation

Fraction	AP activity *% of total activity*	Isoenzyme pattern[a], %	
		K_L	K_I
Nuclear	8	100	0
Mitochondrial	12	100	0
Microsomal	53	100	0
Plasma membrane	20	100	0
Cytoplasm	7	80	20

[a] K_L is liver-like AP in kidney; K_I is intestine-like AP in kidney.

Other parameters used for differentiating AP isoenzymes revealed that intestinal AP and K_I or U_I represent multiple forms of the same isoenzyme. Table 3 shows greater heat stability of both K_I and U_I. K_I showed 45 to 60% and U_I showed 51 to 60% residual activity in contrast to 10 to 25% residual activity of intestinal AP after heating for 90 min up to 56° C. After incubation for 90 min in 5 mM urea, 15% for K_I, 18% for U_I, and 40% residual activity for intestinal AP were found.

K_I and U_I were less inhibited by 5 mM L-phenylalanine (K_I: 43 to 84%, U_I: 60 to 70%) than was the intestinal AP (16 to 23% residual activity). No difference in their electrophoretic migration behavior could be detected when applied to cellulose acetate stripes. To establish whether these differences were caused by butanol extraction, we compared the butanol-extracted intestinal enzyme with that of $\times 100{,}000g$ supernatant of intestinal homogenate. Both intestinal forms could not be differentiated. We also could not find any difference between U_I isolated from the urine of healthy persons, renal pa-

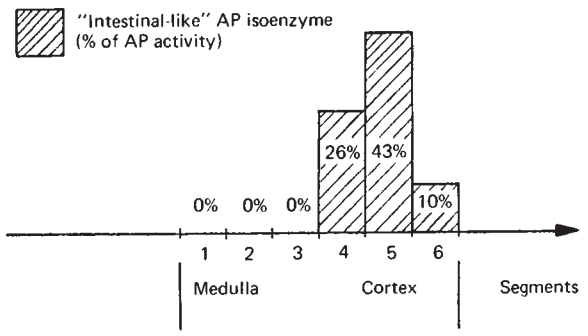

Fig. 3. *Distribution of intestinal-like alkaline phosphatase (AP) isoenzyme K_I in human kidney.*

tients, and subjects undergoing aminoglycoside treatment.

Discussion

By means of an immunotitration technique, the isoenzyme pattern of alkaline phosphatase in the urine of healthy persons has been differentiated quantitatively from that of patients with renal diseases or under application of potentially nephrotoxic drugs. The amount of particle-free AP correlates with the increase of the U_I AP during pathologic renal processes. The elution of sedimentable activity apparently can be correlated with the excretion of renal cell fragments, mainly in urine. In electronic microscopic studies, it could be demonstrated that urine contains microvilli of cell membranes (as complete or vesicle-degenerated microvilli).

Apparently the two AP isoenzymes, the liver (K_L) and the intestinal (K_I) types, extracted from kidney tissue are associated with distinct sites on the tubular epithelia and may represent different developmental stages of the epithelial cells of the proximal convoluted tubules. It has yet to be discussed whether K_I belongs to earlier developmental stages of the proximal tubule. As shown by immunofluorescent microscopy, K_I is located only in single parts of the proximal tubules in the normal renal cortex.

Biochemical characterization of the three intestinal types (intestinal AP, K_I and U_I) evidenced that K_I and U_I can be distinguished as multiple forms of the intestinal AP isoenzyme. The reason for the differences between the K_I and the intestinal

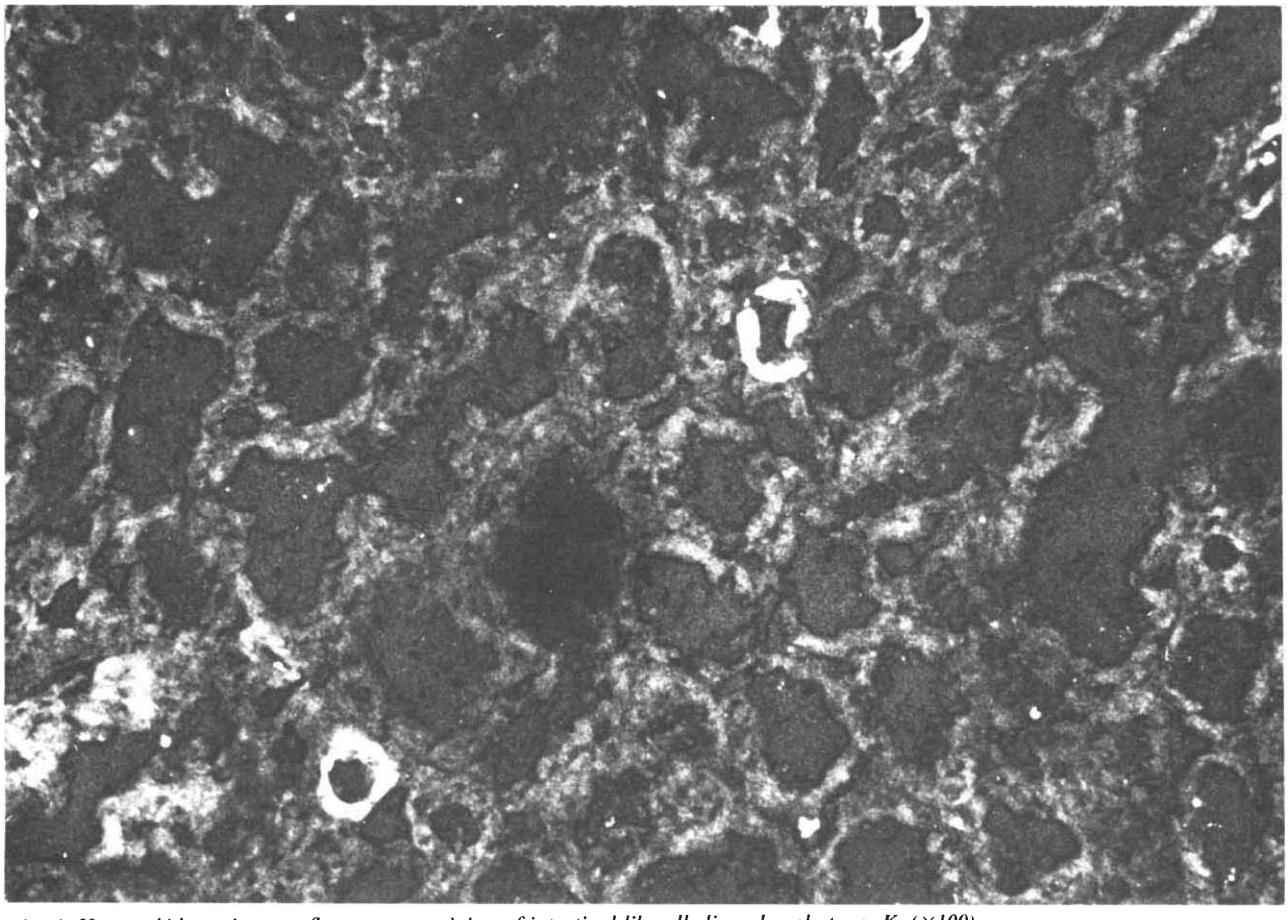

Fig. 4. *Human kidney, immunofluorescent staining of intestinal-like alkaline phosphatase, K_I (×100).*

Table 3. Properties of intestinal alkaline phosphatase (AP) isoenzyme isolated from intestine (I), kidney (K_I), and urine (U_I) of human tissue

Sample[a]	Intracellular localization	$K_m \times 10^{-3}$ M	Molecular weight[b] *daltons*	L-Phe inhibition[c] %	Heat[d] %	Urea[e] %
I	membrane-bound	0.2	124,000	16 to 23	10 to 25	42
K_I	cytoplasmatic	0.5	124,000	43 to 89	45 to 60	15
U_I	particle-free	0.4	124,000	60 to 70	51 to 60	18

[a] Isolated by affinity chromatography
[b] Determined by sucrose density gradient ultracentrifugation
[c] Residual activity after incubation in 5 mM L-Phe for 90 min
[d] Incubation at 56° C for 90 min, residual activity
[e] Incubation in 5 M urea for 90 min, residual activity

AP could be a different carbohydrate content. The distinct behavior on the gel filtration experiments that indicated lower molecular weights for U_I is probably caused by differences in the shape of the molecules. Apparently U_I only originates from the kidney because when isolated from urine in all examined different pathologic states, it exhibited the same properties as K_I.

Previous studies have shown that the AP has to be described as an intrinsic constituent of the luminal plasma membrane in the proximal tubule of the normal human kidney [21, 22]. As alanine aminopeptidase, which is considered as a superficial component of the luminal plasma membrane [23], reached its maximum of output after administration of aminoglycosides earlier than did U_I, elevation of U_I activity indicates probably further tubular damage. During kidney rejection crisis after transplantation the same isoenzyme pattern of urinary AP reflects similar renal damage as that caused by aminoglycosides.

It is likely that under pathologic conditions, more tubular regions containing U_I are developed. As these tubules seem to be damaged more, higher permeability of cellular proteins through the cell membranes leads to an elevated elution of the intestinal-type AP (U_I) in urine. Therefore, intestinal AP isoenzyme, which is the reason for increased AP activity in urine, seems to indicate a lesion of the proximal convoluted tubule and/or of an intensive regeneration of the tubular epithelial cells. An intensive regeneration of these cells occurs certainly in the recovery phase of renal disease and probably accompanies a light or medium renal injury. In all these cases, the increase of the AP in urine appears to be a consequence of an induction of the synthesis as a result of a toxic or inflammatory aggression of the kidney and not only as a result of the loss of the enzyme from the damaged cells.

In preliminary investigations we could exclude that the urinary intestinal AP is leaking from the plasma. During and after application of cytotoxic substances, an increase of serum AP was never observed, and cytoplasmic "intestinal" like AP could never be detected.

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